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Considerations on accuracy, pattern and possible underlying factors of brain microbleeds progression on older adults with absence or mild presence of vascular pathology

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Considerations on accuracy, pattern and possible underlying factors of brain microbleeds progression on older adults with absence or mild presence of vascular pathology

ABSTRACT

Background and Purpose: Visual and computational approaches to assess brain microbleeds (BMBs) on magnetic resonance images (MRI) report inaccuracies up to 30%, but reproducibility and pattern of BMB progression with age haven't been reported. We analyse whether a BMB progression pattern exist, its possible underlying factors, and the influence of inter-observer differences on BMB prevalence and progression, in a sample of older individuals with none or mild vascular pathology.

Participants: We analysed MRI, cognitive, demographic and laboratory data from all individuals from the Alzheimer's Disease (AD) Neuroimaging Initiative database (adni.loni.usc.edu), who had the required sequences for identifying BMBs (i.e. 3DT1- and Axial 2DT2*-weighted sequences) acquired at 3T over 3 consecutive years at the time the database was accessed (January 2016) (n=291).

Methods: BMBs were assessed independently by two observers equally trained and with similar levels of experience following the Brain Observer MicroBleed Scale and published guidelines for their semi-automatic identification. We used the Bland-Altman plot and kappa statistics to analyse inter-observer differences/agreement and analysis of covariance for predictors on their progression.

Results: The number of individuals with BMBs slightly and non-significantly ($p \geq 0.15$) increased across 3 years (22.7% \rightarrow 25.1% \rightarrow 27.5%), as well as the number of BMBs per individual (21% \rightarrow 23% \rightarrow 25% with 1-3 BMBs and 1.7% \rightarrow 2% \rightarrow 2% with ≥ 3 BMBs). The pattern of BMBs prevalence and progression identified by each observer in each cognitive group (normal < early/late mild cognitive impairment (MCI) > AD patients) was similar and non-significant ($p > 0.14$) despite inter-observer differences (1.5 BMB, 95%CI[-3.7 +6.2], presence vs. absence agreement $\kappa=0.543$), which were mainly in the cortex. Serum cholesterol ($p < 0.04$) was the main predictor of change in BMB count between time points but did not predict overall progression.

Conclusions: Even in conditions where MR acquisition and BMBs identification protocols are the same, and observers have similar training and experience, inter-observer differences are present and it is difficult to ascertain their influence in the analysis of BMB progression. BMBs progression was observed in cognitively normal and MCI individuals, but not in AD patients. This should be confirmed in larger studies in more representative populations.

1 INTRODUCTION

Cognitive decline is an ever-increasing challenge to health and social care systems worldwide, due to the growing elderly population. The availability of biomarkers and improved understanding of factors that contribute to heterogeneity in cognitive decline are required to improve diagnosis and prognosis. Small chronic brain haemorrhages occurring with impaired small vessel wall integrity, known as brain microbleeds (BMBs)[1] are commonly found in elderly individuals and have been linked to key hypotheses in the neuro-pathogenesis of the most common form of dementia (i.e. Alzheimer's Disease), namely the 'amyloid cascade'[2] and the 'vascular hypothesis'[3]. However, the clinical effect of BMBs on cognition is an evolving research field[4]. The prevalence of BMBs at different stages within the continuum of cognitive decline, reported by a recent review of multiple studies using different magnetic resonance imaging (MRI) sequences and different magnet strengths[1], are shown in Figure 1.

--- Insert here Figure 1 ---

A further exploratory analysis on the progression pattern of brain mineral deposition, including BMBs, as a differential indicator of cognitive decline in a cross-sectional sample of 200 individuals aged 55-90 years old (58 cognitively normal (CN), 127 with mild cognitive impairment (MCI) and 15 with AD) observed a similar trend, of a higher count in the number of BMBs in individuals with MCI compared to those with AD[5]. Indeed, a number of studies have identified a lack of association between BMBs and cognitive function in AD patients[6,7], indicating that BMBs may not be present in patients with severe AD at all[6] and that cognitive decline in AD may be independent of BMB number[8]. MCI could be manifested on different neurocognitive disorders. However, as MCI is a stage in the continuum of cognitive decline that leads to AD, exploring the manifestations at each stage of this continuum would certainly lead to a better understanding of the AD pathogenesis. Few longitudinal studies have considered the predictive value of BMB detection on the progression of cognitive decline [9,10]. A study[10] found MCI participants with BMBs were twice as likely to progress to dementia than those without BMBs. Another study investigated AD patients and found that patients with BMBs were more cognitively impaired than patients without BMBs, despite having similar disease duration[9]. Although these studies suggest that BMBs may have potential predictive value for progression of cognitive decline, the evidence is limited. The variability in the outcome of the assessment of BMBs when magnetic field strengths and sequence parameters differ is known [11]; and it has been considered the main limitation in the analyses of BMB progression. To the best of our knowledge, and in agreement with a recent review on the reproducibility and variability of MRI markers in cerebral small vessel disease, no study has been found to assess

the reproducibility of any longitudinal measurement for BMBs [11].

In this study, we investigate BMB progression in three consecutive years in groups of individuals with different cognitive status: CN, early MCI (EMCI), late MCI (LMCI), and AD, with the hypothesis that BMB prevalence and count progress in CN and MCI individuals, but not in AD patients. We also evaluate putative risk factors for BMB progression at each time-point and analyse the influence of inter-observer differences on the assessment of BMB progression. Of note, our use of the term ‘progression’ not only refers to whether BMB prevalence (i.e. the proportion of patients with BMBs) and count (i.e. the individual number of BMBs per patient) increase (i.e. progress), but also if decrease (i.e. regress), or remain stable from one year to the next.

2 MATERIALS AND METHODS

Data used in the preparation of this article were obtained from the Alzheimer’s Disease Neuroimaging Initiative (ADNI) database (<http://adni.loni.usc.edu/>). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to evaluate whether serial MRI, positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessments can be combined to measure their progression through the process of normal aging to early mild cognitive impairment (EMCI), to late mild cognitive impairment (LMCI), to dementia or Alzheimer’s disease (AD) (<http://adni.loni.usc.edu/study-design/>).

2.1 Subjects

291 patients were identified from the ADNI database, accessed in January 2016, using the following selection criteria:

- Three consecutive study visits at 12 months apart
- Structural MRI acquisitions at each visit, including T1-weighted (MPRAGE) and T2*-weighted (GRE) sequences, to assess BMBs

The selection was done blind to any clinical, demographical or cognitive data.

2.2 MRI acquisition

The T2*-weighted GRE sequence, which was the primary sequence used for identifying BMBs, was consistently acquired in axial orientation, 2D, with TE/TR = 20/650 ms, flip angle 20°, at 3T, and with an in-plane resolution of 256x256 voxels in all cases. The MPRAGE sequence

was acquired 3D, with TE/TR = 3.16/6.8 ms, flip angle 8°, voxel size of 1x1x1.2 mm³. Although the scanner vendors and coil models were not the same in all centres, all sequence parameters were consistently equal among scanning waves and across centres.

2.3 Image analysis

All (291x3) T2*-weighted (GRE) scans, obtained from each annual visit, were visually assessed by one observer (Observer 1) for BMBs within the software programme MRIcron (www.mricron.com), Ver.12.12.2012. The Brain Observer Microbleed Scale (BOMBS)[12] was used to determine presence, number and location of BMBs. Any black dots initially rated as uncertain were re-rated definitively after considering symmetry, blood vessel anatomy and checking for presence in scans from other visits. Vessel mineralisation was also assessed added by the T1-weighted (MPRAGE) sequence. A cross-sectional subsample of 150, and a longitudinal subsample of 20 patients, both randomly selected, were equally assessed by a second observer (Observer 2) with a similar level of experience and following the same guidelines. All assessments were done by each observer blind to each other and to any clinical, demographic or cognitive data. The time difference between each observer's assessments was 1 year. A third observer checked each observer's results at the time of their respective assessments, simultaneously viewing all sequences from the three yearly scans of the same patient, to guarantee consistency in the output and decide on "uncertain" cases.

2.4 Clinical data

Relevant clinical information for all patients, selected based on clinical plausibility and/or previous research, was extracted from the ADNI database as per Table 1.

-- Insert Table 1 here --

2.5 Statistical analyses

Statistical tests were performed using the Statistical Package for Social Sciences (SPSS) Version 24 and MATLAB R2015a.

2.5.1 Analyses of progression of BMBs

The Mann-Whitney U test was used to determine differences in the distribution of BMB prevalence (i.e. the proportion of patients with BMBs) between cognitive groups each year and the median number of BMBs in each cognitive group each year to evaluate BMB progression (as defined previously).

2.5.2 Analyses of risk factors for BMB progression

One-way analyses of covariance (ANCOVA) were performed to evaluate candidate variables

(clinical data) associated with potential change in BMB count at each time-point. Since BMB counts were obtained at three time-points (year one (Y1), year two (Y2) and year three (Y3)) evaluation was performed for potential change from Y1 to Y2, Y2 to Y3 and Y1 to Y3. Candidate variables were selected a priori based on clinical plausibility and/or previous research (Table 1). Data exploration suggested three ANCOVA analyses for bio-specimens (ANCOVA 1), brain measurements (ANCOVA 2) and cognition (ANCOVA 3) should be performed at each time-point. Prior to conducting ANCOVAs, collinearity was assessed through Belsley collinearity diagnostics[13] and any covariates found to have statistically significant associations with other covariates included in each ANCOVA were excluded. Independence between each covariate and the independent variable and homogeneity of regression slope assumptions were also tested and any statistically significant associations excluded from analysis. Finally, principal component analysis (PCA) was performed prior to conducting the cognition ANCOVA to restrict cognitive test scores to four principal components that explained 78.38% of the variance. Finally, statistically significant predictors for potential change in BMB count at each time-point from the ANCOVAs were then used to calculate odds ratios to determine whether each predictor was a factor for BMB progression, i.e. an increase in BMB count from Y1 to Y2, Y2 to Y3 or Y1 to Y3. For this, significant predictors from ANCOVAs were dichotomised appropriately; presence or absence for binary factors and clinical criterion used for continuous factors.

2.5.3 Analyses of inter-observer variability

BMB counts reported by each observer for the subsample of 150 patients (cross-sectional subsample) were used to assess inter-observer variability on BMB prevalence per cognitive group. A Bland-Altman plot[14] was constructed to evaluate inter-observer differences. The Mann-Whitney U test was used to determine whether differences in BMB prevalence between cognitive groups were significant. Cohen's kappa for BMB presence/absence and for BMB count were also calculated, using <http://vassarstats.net/kappa.html> (Copyright Richard Lowry 2001-2015) and the MATLAB function kappa, ver. 1.3 by Giuseppe Cardillo, downloadable from <https://uk.mathworks.com/matlabcentral/fileexchange/15365-cohen-s-kappa>.

BMB counts reported by each observer for the subsample of 20 patients (longitudinal subsample) were used to assess inter-observer variability on BMB progression per cognitive group. The Mann-Whitney U test was used to determine significance in the differences in average BMB prevalence between cognitive groups. The median number of BMBs reported by each observer, in each cognitive group each year, was also calculated.

Differences in BMB counts reported by each observer were further investigated. Reported BMB location was considered to ascertain whether either observer was consistently identifying more BMBs in a particular location. An experienced observer also re-assessed scans where

inter-observer differences were reported to investigate factors that may have confounded BMB assessments.

3 RESULTS

In general, BMBs progression remained stable across the three years in all cognitive groups despite BMBs count increase was observed in some CN and MCI individuals. It resulted unfeasible to determine whether BMB progression in such a short time could be considered an indicator of cognitive decline, as detailed below.

3.1 Sample characteristics

The descriptive statistics for each group's baseline characteristics and number of BMBs counted by Observer 1 are presented in Table 2. Prevalence of BMBs in the sample slightly increased over the three years (22.68% → 25.08% → 27.49%) as did the individual BMB count per patient (20.96% → 23.02% → 25.43%) with 1-3 BMBs and (1.72% → 2.06% → 2.06%) with ≥3 BMBs. This slight increase was observed in CN and MCI individuals, but not in AD patients.

-- Insert Table 2 here --

Of note, 93/201 patients that comprised this sample were part of the cross-sectional sample analysed in [5].

3.2 BMB progression pattern in the total sample

BMBs were identified in 66 patients in Y1, 73 patients in Y2, and 80 patients in Y3. BMBs were found in 25/98, 26/98, 28/98 CN, 24/100, 26/100, 26/100 EMCI, 13/76, 17/76, 22/76 LMCI and 4/17, 4/17, 4/17 AD patients in Y1, Y2 and Y3 respectively (Figure 2).

--Insert Figure 2 here --

BMB prevalence overall was higher in the CN group than in the EMCI ($p_{Y1}=0.629$, $p_{Y2}=0.721$, $p_{Y3}=0.6464$), LMCI ($p_{Y1}=0.160$, $p_{Y2}=0.431$, $p_{Y3}=0.928$) and AD groups ($p_{Y1}=0.996$, $p_{Y2}=0.935$, $p_{Y3}=0.928$) each year, but these group differences were non-significant. The median number of BMBs per-patient was greater in AD (2.5,2.5,2.5) compared to CN (1,1,1), EMCI (1,1,2) and LMCI (1,1,1) groups, but again, these group differences were all non-significant.

In general, from Y1 to Y2, BMB count increased in 16/291 individuals and decreased in 8/291 individuals. From Y2 to Y3 BMB count increased in 21/291 individuals and decreased in 7/291 individuals. The median increase/decrease counted was 1 BMB. Only 4/291 individuals experienced a consistent yearly increase in the number of BMBs.

3.3 Factors for BMB progression

Statistically significant predictors for potential change in BMB count at each time-point identified by ANCOVA analyses are shown in Table 3. Total cholesterol was the main predictor ($B=0.002$, $p<0.04$) when included in ANCOVA 1. Years in education ($B=-0.029$, $p=0.028$) and family history of dementia ($B=-0.102$, $p=0.028$) were also found to be predictors when included. Family history of AD ($B=0.060$, $p=0.016$) and endocrine-metabolic risk factors ($B=0.10$, $p=0.044$) were found to predict potential change in BMB count from Y1 to Y2 in ANCOVAs 2 and 3 respectively. However, as Table 3 shows, significant predictors for potential change in BMB count at each time-point could not be considered significant **factors that could potentially increase the likelihood of BMB to occur**.

--Insert Table 3 here--

3.5 Analyses of inter-observer variation on BMB progression pattern assessment

3.5.1 Cross-sectional inter-observer differences

Figure 3 (a) shows the Bland-Altman plot. **Both observers had perfect agreement on 68/150 cases**. The average difference of BMB count between observers is 1.47, suggesting a bias towards Observer 2 reporting a higher count. Inter-observer differences increased with the increase in the number of BMBs counted. Observer 2 had a tendency to report a higher BMB count in the cortex, a likely explanation for the bias and increasing difference. Observer 1 found BMBs present in 9/50 CN, 15/54 EMCI, 6/38 LMCI, and 1/8 AD patients (Figure 3 (b)) and reported a BMB prevalence lower in CN, than EMCI patients ($p=0.252$). However, BMB prevalence was higher in CN, than LMCI ($p=0.797$) and AD patients ($p=0.668$). These differences were also non-significant. Observer 2 found BMBs present in 22/50 CN, 28/54 EMCI, 21/38 LMCI, and 4/8 AD patients (Figure 3 (b)) and reported a BMB prevalence lower in CN, than EMCI ($p=0.147$), LMCI ($p=0.175$) and AD patients ($p=0.719$) although these differences were non-significant. While the median number of BMBs found by Observer 1 was slightly greater in the LMCI group (1.7) than the CN (1), EMCI (1) and AD (1) groups, the median number of BMBs found by Observer 2 was slightly greater in the EMCI group (3.5) than the AD (2.5), CN (2) and LMCI (2) groups.

--Insert Figure 3 here--

The overall agreement between Observers 1 and 2, of having vs. not having BMBs was $\kappa=0.543$. However, there was only a slight agreement between both observers in the exact number of BMB counted: unweighted Cohen's kappa $\kappa = 0.058 [-0.077 \ 0.194]$, with a maximum possible kappa given the marginal frequencies $\kappa= 0.424$.

3.5.2 Longitudinal inter-observer differences

Observer 1 found BMBs present in 2/3 CN, 2/12 EMCI and 2/5 LMCI patients (Figure 4) and reported an average BMB prevalence higher in CN than EMCI ($p=0.115$) and LMCI patients ($p=0.751$), over each year although these differences were non-significant. Observer 2 found BMBs present in 1/3 CN, 5/12 EMCI and 3/5 LMCI patients (Figure 4) and reported an average BMB prevalence higher in LMCI compared to EMCI ($p=0.677$) and CN patients ($p=0.863$), over each year. However, the differences in BMB prevalence between cognitive groups were also non-significant. The median number of BMBs identified by each observer in each cognitive group per year is detailed in Table 4. Both observers identified BMBs progress in the CN group each year. Similarly, for EMCI patients, both observers found BMBs progress from Y1 to Y2 and then regress from Y2 to Y3. For LMCI patients, both observers identify BMBs progress from Y1 to Y2, but Observer 2 reported a regression from Y2 to Y3 whereas Observer 1 reported a progression.

--Insert Figure 4 and Table 4 here --

3.5.3 Factors that affected inter-observer reliability and location of the inter-observer differences

Observer 2 had a tendency to report a higher BMB count in the basal ganglia in the subsample of 20, and in the cortex in the subsample of 150. Inter-observer differences were mainly found in these two locations (i.e. the basal ganglia and cortex). However, in the subsample of 150 median differences of 1 BMB were found in the grey/white matter junction (3 cases), brain stem (6 cases), thalami (2 cases), cerebellum (8 cases) and subcortical white matter (3 cases). Vessel calcifications (Figure 5a), basal ganglia mineralisation (Figure 5b) and reduced cortical thickness (Figure 5c) hampered identification of 'true' BMBs and hence affected inter-observer reliability.

--Insert Figure 5 here --

4 DISCUSSION

Our findings suggest that the number of BMBs may increase in cognitively normal and mild cognitive impaired individuals but not in Alzheimer's disease patients. However, a progression pattern was not present, and none of the factors reported up-to-date to be associated with BMB prevalence appeared to predict these results, although total serum cholesterol was identified as the main potential predictor of the change in BMB count at each time-point. Despite equal assessment conditions, inter-observer variations can be considered problematic

in the assessment of BMB progression: their variation seems to have little influence in the analysis of BMB progression in the sample, not so in individual cases.

4.1 Progression pattern of BMBs

Contrary to our hypothesis, progression of BMBs in CN and MCI groups of individuals could not be ascertained, as it was observed only on a small proportion of individuals from each group. BMB prevalence remained stable in all AD (22.76%) patients as hypothesised. However, the AD group of patients had higher median number of BMBs. The lack of progression maybe due to the low prevalence of vascular risk factors in AD patients compared to the other groups of individuals. Despite considerable heterogeneity within existing literature, our findings contrast with the general trend reported[1]. For example, the Rotterdam study investigated elderly individuals without dementia and reported progression in BMB prevalence from 24.4% at baseline to 28% after three years[15]. Although a percentage of CN individuals with BMBs in our study showed similar progression, increasing from 25.51% to 28.57% three years later, in our case this increase was non-significant in the overall sample. Possible explanations for this discrepancy include differences in: sample sizes, study population characteristics, MRI sequences and imaging parameter choices, inter-observer variation and/or observer experience. Many studies in [1] used small sample sizes[16,17,10,18], particularly for CN controls, which may not be representative of BMB prevalence per cognitive group. MRI sequence and imaging parameter choice can also affect BMB identification[19] with susceptibility-weighted images enhancing the susceptibility-related contrasts, and higher magnetic fields increasing the contrast-to-noise ratio and the BMBs size. Different study sample characteristics may also have influenced BMB prevalence. Our sample is not representative of the general population, as individuals that have baseline MRI scans with evidence of infarction or other focal lesions, presence of multiple lacunes or lacunes in a critical memory structure, and/or other brain structural abnormalities are excluded from ADNI, hence only individuals with absence or mild presence of vascular pathology are included. Moreover, in our sample, the mean age in the CN group (mean 73.60 years old) was slightly higher than the MCI groups (70.16 & 70.68 years respectively) and the proportion of CN individuals with two cardiovascular risk factors (29.59%) was also greater than for MCI (26.00% & 21.05%) and AD (5.88%) patients. BMB prevalence is known to increase with age and cardiovascular risks[20,21,22]. Finally, accurate BMB identification is crucial to reporting BMB prevalence, and to our knowledge, this has not been analysed in any of the previous longitudinal studies of BMB progression.

4.2 Risk factors for BMB progression

No specific clinical risk factors predicted BMB progression. The association of specific clinical

risk factors with BMBs is somewhat conflicting in existing literature. E.g. A study[21] found low total cholesterol to be associated with BMB prevalence, whereas this study found elevated total cholesterol to predict potential change in BMB count from Y1-Y2 and Y1-Y3. This latter finding is more consistent with that reported in a study of dementia patients, with BMB prevalence increasing with hyperlipidaemia and with MCI patients with hyperlipidaemia having increased BMB counts[22]. Similarly, conflicting findings have been reported for endocrine-metabolic factors, specifically diabetes[22,15]. There is a dearth of information on factors and neuropathological markers of cognitive decline and dementia progression, which include years in education, family history of AD and BMBs ([23,24,25] for citing some). The present study is specific to factors that contribute to potential change in BMB count and therefore not directly comparable to research on cognitive decline per se.

Odds ratios were small with confidence intervals overlapping one. Predictors derived from ANCOVA models may not have translated into significant odds ratios due to sample size and/or small effect size of putative risk factors, and/or dichotomisation. Progression was only reported in 18 patients from Y1-Y2, 23 patients from Y2-Y3 and 28 patients from Y1-Y3. Such sample sizes may have been too small to produce significant findings. Furthermore, the predictors determined by ANCOVA had to be dichotomised to calculate odds ratios. Such dichotomisation may have resulted in loss of statistical power. Indeed, two studies investigating risk factors associated with BMBs used much larger sample sizes and logistic regression models to avoid limitations associated with dichotomisation[20,21]. They still reported odds ratios and confidence intervals quite close to (and often overlapping) one. The progression of other markers of cerebral small vessel disease like white matter hyperintensities could predict the progression of BMBs. However, only visual ratings of white matter hyperintensities and perivascular spaces at Y1 were available for the analyses. Future longitudinal studies require larger sample sizes, quantitative measures of small vessel disease indicators at each time point and/or alternative statistical approaches to fully investigate risk factors for BMB progression.

4.3 Influence of inter-observer variation on the assessment of BMB progression

Variation in inter-observer differences was observed. Bland-Altman analysis found a bias towards Observer 2 reporting a higher BMB count, with an increased difference as BMB count increased. However, the analysis of the overall BMB progression in the sample yielded similar results for both observers. Despite recognition of large ranges of inter-observer reliability within existing literature[12], there are no guidelines on what constitutes clinically acceptable bias/limits of agreement. Most studies have reported kappa statistics; however, this parameter is limited in interpreting extent of agreement, with the quantitative value being qualitatively

described as slight, fair, moderate etc. For consistency in the way results are reported and to allow comparability with other studies, we also report kappa statistics. Bland-Altman methodology, though, is more appropriate to parametric data, permitting the meaningfulness of change to be determined within the context of the measurement scale (i.e. the 95% limits of agreement are placed in 'test score' units in contrast to kappa).

Factors found to affect inter-observer reliability have previously been reported as those confounding BMB detection[26,27,11]. Basal ganglia mineralisation is likely to explain Observer 2's tendency to report a higher BMB count in the basal ganglia, and vessel calcifications and reduced cortical thickness, hampering differentiation between BMBs and vessel calcification, explaining the higher BMB count reported for the cortex. Previous study on 100 CN community-dwelling individuals aged 73 years showed a positive and strong correlation and considerable overlap between calcified regions and other regions with iron accumulation[28] in its different forms[29]. Others have suggested a shared pathway for the age-related deposition of both minerals in the brain, occasionally attributed to the primary accumulation of one followed by the other[30], or to a shared transport mechanism that manifest in the deposition of both minerals in the vicinities of the blood vessels[31]. These may partly explain why inter-observer differences increased with the increase of the BMB count and the consistency in the overall progression pattern of BMBs found by both observers despite the influence that the inter-observer differences had on the assessment of the BMB progression in individual cases. Future studies should seek to reduce inter-observer variation and there should be a clinical consensus on acceptable bias/limits of agreement.

4.4 Strengths and limitations

To the best of our knowledge, this is the first study that assesses the reproducibility of longitudinal measurements of BMBs, as such contributing to the emerging field of research on BMBs as potential pathological markers within the spectrum of cognitive decline. Strengths of this study also include the large number of individuals assessed under the same MRI protocol using well-validated BMB guidelines and the wide variety of relevant participant data available. The observers were blinded to clinical data, including cognitive status, throughout image analysis.

A limitation of this study is the relatively small AD group. Longitudinal study designs and the nature of AD may impede obtaining comparatively sized AD groups[32]; however, future studies should try to secure larger AD populations to facilitate valid comparisons. This study was also limited by the inclusion/exclusion criteria of the ADNI database, a cohort not entirely representative of the general population[33]. Individuals with substantial small vessel disease

are excluded from ADNI and therefore AD patients are likely to be more genetically prone to the disease. Clinical study data included was also restricted to available ADNI database parameters collected across multiple sites. Although a general protocol is in place, variations in techniques and data collection methodologies are likely. The location of BMBs was not statistically analysed in this study. Lobar BMBs have been previously found to be associated with longitudinal cognitive decline, whereas BMBs located in deep grey matter and infratentorial brain revealed no significant associations[34]. BMBs in *all* regions were included in this study, potentially diluting findings. A further limitation is the failure to apply Bonferroni correction to the ANCOVA models that would have resulted in a more stringent criterion for statistical significance. Finally, intra-observer variation was not considered here due to time constraints; such measurements are also important to determine, although might reasonably be expected to be less than inter-observer variations.

5 CONCLUSIONS AND FUTURE DIRECTIONS

BMB prevalence and count appeared to progress in CN and MCI individuals but not AD patients; however, differences between cognitive groups were non-significant and the median number of BMBs tended to remain stable in each group over time. No significant progression pattern was found in the sample analysed, although as noted above this sample may not be representative of the general ageing population. Although total cholesterol, years in education, family history of dementia and AD, and endocrine-metabolic risk factors were weakly predictive of potential change in BMB count at certain time-points, they were not significant risk factors for BMB progression. Finally, despite equal conditions, inter-observer differences were found and inter-observer variation *is* likely to have influenced BMB progression pattern assessment. Therefore, this report cannot exclude BMB progression in AD patients.

While this study highlights the importance of reporting inter-observer variation, future studies should seek to reduce inter-observer differences. Similar inter-observer variation analyses could be performed for computational rather than visual assessments of BMBs, to determine whether computational assessment methods are more reliable. Repeating this study in a few years may yield different results as the cognitive status of individuals enrolled in ADNI is likely to advance along the continuum of cognitive decline and more patients are likely to have had three consecutive study visits, minimising AD group limitations. A similar study could also be performed in a larger sample of patients, adjusting for baseline characteristics, and across a wider spectrum of cognitive decline. **Also, analysing a wider interval between assessments and/or increasing the number of yearly assessments (i.e. analysing progression in 4 or more years) would be clinically important.** Analysis of BMB location may also be informative. Such

a study is likely to provide useful information on BMB progression and associated risk factors, confirming or otherwise the potentially valuable role of BMBs as a differential indicator of cognitive decline.

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TABLES

Table 1. Clinical data downloaded from the ADNI database and clinical plausibility and/or previous research for selection

Clinical Data Obtained	Clinical Plausibility & Previous Research
General Information <ul style="list-style-type: none"> • Diagnosis at Baseline (CN, EMCI, LMCI or AD) • Age at Baseline • Gender • Number of years in Education • Race • Marital Status • APOE4 Genotype 	(Brayne et al., 2010, Charidimou et al., 2013, Copenhaver et al., 2008, Helmer et al., 1999, Jeerakathil et al., 2004, Lee et al., 2011, Poels et al., 2010, Poels et al., 2011, Romero et al., 2014, Shams et al., 2015, Sveinbjornsdottir et al., 2008, Tsushima et al., 2003, van Gelder et al., 2006, Wiegman et al., 2014, Wu et al., 2014)
Baseline Cognitive Test Scores <ul style="list-style-type: none"> • Clinical Dementia Rating Scale • Cognitive Subscale of the Alzheimer's Disease Assessment Scale (11 and 13) • Mini Mental State Examination • Auditory Verbal Learning Test • Activities of Daily Living • Montreal Cognitive Assessment • Everyday Cognition (both patient and study partner) 	(Wu et al., 2014) Post meta-analysis relevant studies; (Hilal et al., 2014, Shams et al., 2015, Yamashiro et al., 2014)
Baseline Brain Measurements <ul style="list-style-type: none"> • Ventricles • Hippocampus • Whole Brain • Entorhinal Cortex • Fusiform Gyrus • Middle Temporal Gyrus 	(Bilello et al., 2015, Chowdhury et al., 2011, Henneman et al., 2009, Samuraki et al., 2015, Su et al., 2016)

<ul style="list-style-type: none"> • Intracranial Volume (ICV) • Fazekas visual ratings • Perivascular spaces visual ratings <p>N.B. All baseline brain measurements (i.e. not the visual ratings) were standardised by ICV measurements for statistical analysis.</p>	
<p>Medical and Family History</p> <ul style="list-style-type: none"> • Systolic and Diastolic Blood Pressure (mmHg) at baseline • Seated Pulse Rate (per minute) at baseline • Body Mass Index at screening • Past Medical History of Cardiovascular Risk Factors (Cardiovascular Disease and Smoking) and Endocrine-Metabolic Risk Factors at baseline • Family History of Dementia and Alzheimer's Disease at baseline 	<p>(Charidimou et al., 2013, Copenhaver et al., 2008, Gómez-Tortosa et al., 2012, Jeerakathil et al., 2004, Lee et al., 2011, Poels et al., 2010, Poels et al., 2011, Romero et al., 2014, Scarabino et al., 2016, Shams et al., 2015, Sveinbjornsdottir et al., 2008, Tsushima et al., 2003, Wiegman et al., 2014, Wu et al., 2014)</p>
<p>Baseline Subject Characteristics</p> <ul style="list-style-type: none"> • Work History • Retirement Status 	<p>(Andel et al., 2015, Leist et al., 2013, Rijs et al., 2015)</p>
<p>Baseline Bio-specimen Measurements</p> <ul style="list-style-type: none"> • Red Blood Cells ($\times 10^{12}/L$) • Haemoglobin (g/dL) • Haematocrit (%) • Triglycerides (GPO) (mg/dL) • Cholesterol (mg/dL) • Serum Glucose (mg/dL) • Creatinine (mg/dL) • Vitamin B12 (pg/mL) 	<p>(Andro et al., 2013, Charidimou et al., 2013, Clarke et al., 2007, Copenhaver et al., 2008, Jeerakathil et al., 2004, Lee et al., 2011, Poels et al., 2010, Poels et al., 2011, Romero et al., 2014, Shams et al., 2015, Sveinbjornsdottir et al., 2008, Taniguchi et al., 2014, Tsushima et al., 2003, Wiegman et al., 2014, Wu et al., 2014, Wieberdink et al., 2011)</p>

Note: Past medical history of Cardiovascular Risk Factors refers to smoking, other risk factors mentioned in the participant's medical history, and previous medical reports of having (or not) any cardiovascular disease. The latter referred to/included the presence of coronary or peripheral artery disease, mild stroke, hypertensive or rheumatic heart disease, cardiomyopathy, carditis, heart arrhythmia, or thromboembolic disease. The most common risk factors described in the participant's medical history are hypertension and hypercholesterolaemia.

Table 2. Descriptive statistics of baseline characteristics for the total sample and by cognitive group. For convenience of compact tabular presentation percentages have been rounded to the nearest whole number (elsewhere in the text percentages are quoted to two decimal places as appropriate to the sample size).

Variable type	Total (n=291)	CN (n=98)	EMCI (n=100)	LMCI (n=76)	AD (n=17)
<i>Age (years) [mean (SD)]</i>	72 (7.27)	74 (6.16)	70 (6.80)	71 (8.25)	74 (8.45)
<i>Gender [% (n)]</i>					
Male	53 (155)	50 (49)	59 (59)	47 (36)	65 (11)
Female	47 (136)	50 (49)	41 (41)	53 (40)	35 (6)
<i>Race [% (n)]</i>					
Asian	1 (4)	2 (2)	2 (2)	0 (0)	0 (0)
Native Hawaiian or Other Pacific Islander	0 (1)	0 (0)	1 (1)	0 (0)	0 (0)
Black or African American	3 (10)	7 (7)	0 (0)	4 (3)	0 (0)
White	93 (270)	90 (88)	94 (94)	95 (72)	94 (16)
More than one race	2 (6)	1 (1)	3 (3)	1 (1)	6 (1)
<i>Education (number of years) [mean (SD)]</i>	17 (2.49)	17 (2.24)	16 (2.73)	17 (2.39)	16 (2.73)
<i>Marital Status [% (n)]</i>					
Married	74 (214)	68 (67)	78 (78)	71 (54)	88 (15)
Widowed	9 (27)	12 (12)	3 (3)	14 (11)	6 (1)
Divorced	13 (39)	15 (15)	14 (14)	12 (9)	6 (1)
Never married	3 (10)	4 (4)	4 (4)	3 (2)	0 (0)
Unknown	0 (1)	0 (0)	1 (1)	0 (0)	0 (0)
<i>APOE ε4 genotype [% (n)]</i>					
No copies of APOE ε4	58 (169)	76 (74)	53 (53)	53 (40)	12 (2)
One copy of APOE ε4	34 (98)	21 (21)	40 (40)	36 (27)	59 (10)
Two copies of APOE ε4	8 (24)	3 (3)	7 (7)	12 (9)	29 (5)
<i>Key Cognitive Test Scores [mean(SD)]</i>					
Clinical Dementia Rating Scale	1 (1.29)	0 (0.14)	1 (0.76)	2 (1.04)	4 (1.79)
Alzheimer's Disease Assessment Scale 13 - Cognitive	14 (7.18)	9 (4.04)	12 (4.78)	18 (6.41)	29 (7.32)
Mini Mental State Examination	28 (2.23)	29 (1.23)	29 (1.67)	28 (1.78)	22 (1.67)
Activities of Daily Living	2 (4.00)	0 (0.53)	2 (3.17)	3 (4.08)	10 (6.93)
	*(n=263)	*(n=92)	*(n=91)	*(n=65)	*(n=15)

<i>Brain Measurements (ml)</i>					
[median (IQR)]					
Ventricles	30.62 (22.98)	29.77 (21.89)	29.11 (23.79)	30.51 (29.10)	38.59 (15.44)
Hippocampus	7.43 (1.53)	7.69 (1.33)	7.63 (1.47)	6.91(1.56)	5.65 (1.48)
Whole Brain	1055.66 (145.62)	1052.20 (127.64)	1083.26 (129.57)	1030.24 (161.40)	970.35 (160.32)
Entorhinal Cortex	3.76 (0.91)	3.84 (0.80)	3.92 (0.70)	3.43 (0.99)	2.88 (0.81)
Fusiform Gyrus	18.15 (3.43)	18.26 (3.18)	18.90 (3.51)	17.41 (2.24)	16.07 (2.49)
Middle Temporal Gyrus	20.06 (3.70)	20.22 (3.66)	21.06 (3.07)	19.36 (3.20)	17.64 (2.10)
Intracranial Volume	1516.42 (210.06)	1517.43 (227.86)	1526.83 (196.76)	1498.17 (196.00)	1515.68 (226.14)
<i>Vital Signs [mean (SD)]</i>					
Diastolic Blood Pressure (mmHg)	74 (9.95)	74 (9.98)	74 (10.09)	74 (10.24)	73 (8.27)
Systolic Blood Pressure (mmHg)	133 (17.52)	135 (16.77)	131 (18.31)	133 (17.43)	131 (17.73)
Seated Pulse Rate (per minute)	65 (10.97)	65 (11.92)	65 (9.97)	65 (11.48)	62 (9.09)
	*(n=289)	*(n=96)			
<i>BMI [mean (SD)]</i>	27 (5.26)	27 (5.81)	27 (5.00)	28 (5.17)	25 (3.33)
<i>Past Medical History of Cardiovascular (CV) Risk Factors (CV disease and Smoking) [% (n)]</i>					
No CV risk factors	14 (42)	12 (12)	13 (13)	16 (12)	29 (5)
One CV risk factor	61 (177)	58 (57)	61 (61)	63 (48)	65 (11)
Two CV risk factors	25 (72)	30 (29)	26 (26)	21 (16)	6 (1)
<i>Past Medical History of Endocrine-Metabolic Risk Factors [% (n)]</i>					
No	57 (165)	58 (57)	59 (59)	55 (42)	41 (7)
Yes	43 (126)	42 (41)	41 (41)	45 (34)	59 (10)
<i>Family History of Dementia [% (n)]</i>					
Neither parent	40 (115)	49 (48)	33 (33)	34 (26)	47 (8)
One parent	47 (138)	43 (42)	52 (52)	50 (38)	35 (6)
Both parents	5 (14)	4 (4)	5 (5)	7 (5)	0 (0)

Don't know	8 (24)	4 (4)	10 (10)	9 (7)	18 (3)
<i>Family History of Alzheimer's Disease [% (n)]</i>					
Neither parent	45 (132)	55 (54)	37 (37)	42 (32)	53 (9)
One parent	35 (101)	38 (37)	41 (41)	26 (20)	18 (3)
Both parents	1 (4)	2 (2)	1 (1)	1 (1)	0 (0)
Don't know	19 (54)	5 (5)	21 (21)	30 (23)	29 (5)
	*(n=289)		*(n=98)		
<i>Work History classified by 'Social Class based on Occupation' (OPCS, 1991) [% (n)]</i>					
Professional occupation	13 (39)	13 (13)	10 (10)	18 (14)	12 (2)
Intermediate occupation	55 (159)	56 (55)	53 (52)	57 (43)	53 (9)
Non-manual skilled occupation	18 (53)	19 (19)	20 (20)	13 (10)	24 (4)
Manual skilled occupation	5 (14)	1 (1)	9 (9)	5 (4)	0 (0)
Partly skilled occupation	2 (6)	2 (2)	3 (3)	0 (0)	6 (1)
Unskilled occupation	0 (1)	1 (1)	0 (0)	0 (0)	0 (0)
Homemaker/Mother/Volunteer	6 (17)	7 (7)	4 (4)	7 (5)	6 (1)
<i>Retirement Status [% (n)]</i>					
Retired	74 (214)	95 (74)	67 (67)	75 (57)	94 (16)
Not Retired	23 (68)	20 (20)	32 (32)	20 (15)	6 (1)
Not Applicable	3 (9)	4 (4)	1 (1)	5 (4)	0 (0)
	*(n=289)	*(n=97)		*(n=75)	
<i>Bio-specimen Measurements</i>					
Red Blood Cells (x10 ¹² /L) [mean (SD)]	4.6 (0.44)	4.5 (0.40)	4.5 (0.50)	4.6 (0.40)	4.7 (0.40)
Haemoglobin (g/dL) [mean (SD)]	13.9 (1.40)	13.8 (1.40)	14.0 (1.56)	13.9 (1.17)	14.6 (1.19)
Haematocrit (%) [mean (SD)]	41 (4.04)	41 (3.97)	41 (4.52)	41 (3.43)	44 (3.48)
Triglycerides (GPO) (mg/dL) [median (IQR)]	111 (87)	103 (71)	121 (90.3)	112 (94.5)	103 (92)
Cholesterol (mg/dL) [mean (SD)]	190(39.42)	187 (36.42)	190 (43.56)	192 (32.25)	203 (56.82)
Serum Glucose (mg/dL) [median (IQR)]	96 (16)	96 (15)	96 (17)	97 (19)	95 (19)
Creatinine (mg/dL) [mean (SD)]	0.99 (0.23)	1.02 (0.25)	0.96 (0.20)	0.97 (0.23)	1.06 (0.21)

Vitamin B12 (pg/mL) [median (IQR)]	468 (359)	455 (362)	470 (402)	470 (295)	545 (362)
<i>BMB Count [% (n)]</i>					
Year 1					
0	77 (225)	74 (73)	76 (76)	83 (63)	76 (13)
1-3	21 (61)	26 (25)	21 (21)	17 (13)	12 (2)
≥3	2 (5)	0 (0)	3 (3)	0 (0)	12 (2)
Year 2					
0	75 (218)	73 (72)	74 (74)	78 (59)	76 (13)
1-3	23 (67)	27 (26)	22 (22)	22 (17)	12 (2)
≥3	2 (6)	0 (0)	4 (4)	0 (0)	12 (2)
Year 3					
0	73 (211)	71 (70)	74 (74)	71 (54)	76 (13)
1-3	25 (74)	28 (27)	22 (22)	29 (22)	18 (3)
≥3	2 (6)	1 (1)	4 (4)	0 (0)	6 (1)

Note: *(n): Amended sample size where data unavailable.

Table 3. Statistically significant predictors for potential change in BMB count at each time-point, and odds ratios (with 95% confidence intervals) of whether the statistically significant predictors are or not risk factors for BMB progression.

ANCOVA 1 – Biospecimen Measurements & Other	
<i>BMBY1 → BMBY2</i>	<i>Cholesterol (B=0.002, SE=<0.001, p=0.0097) (OR=1.36, CI=[0.52-3.56])</i>
<i>BMBY2 → BMBY3</i>	<i>Number of years in Education (B=-0.029, SE=0.013, p=0.028) (OR=0.60, CI=[0.13-2.64])</i>
<i>BMBY1 → BMBY3</i>	<i>Cholesterol (B=0.002, SE=<0.001, p=0.044) (OR=1.28, CI=[0.58-2.82]) Family History of Dementia (B=-0.10, SE=0.046, p=0.028) (OR=1.01, CI=[0.46-2.23])</i>
ANCOVA 2 – Brain Measurements & Other	
<i>BMBY1 → BMBY2</i>	<i>Family History of AD (B=0.060, SE=0.025, p=0.016) (OR=1.47, CI=[0.52-4.20])</i>
	-
<i>BMBY2 → BMBY3</i>	
	-
<i>BMBY1 → BMBY3</i>	
ANCOVA 3 – Cognition & Other	
<i>BMBY1 → BMBY2</i>	<i>Endocrine-Metabolic Risk Factors (B=0.10, SE=0.050, p=0.044) (OR=1.33, CI=[0.51-3.46])</i>
	-
<i>BMBY2 → BMBY3</i>	
	-
<i>BMBY1 → BMBY3</i>	

Note: BMBY1: BMB count in Y1 (first visit) brain scan, BMBY2: BMB count in Y2 (second visit) brain scan, BMBY3: BMB count in Y3 (third visit) brain scan, B: un-standardised regression coefficients, SE: standard error, p: p-values, OR: odds ratio, CI: 95% confidence interval.

Table 4. Median number of BMBs identified by each observer in each cognitive group each year on a subsample of 20 patients.

	Observer 1 Median Number of BMBs			Observer 2 Median Number of BMBs		
	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
CN	1	1	1	3	5	6
EMCI	2.5	2.5	2.5	5	6	5
LMCI	1.5	1.5	1.5	2	3	2

Note: CN: cognitively normal, EMCI: early mild cognitive impairment, LMCI: late mild cognitive impairment

FIGURES

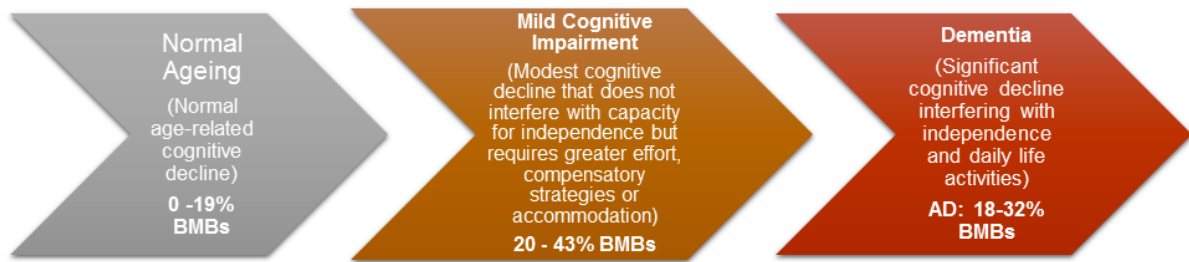


Figure 1. Schematic representation of the continuum of cognitive decline: from cognitively normal to dementia, as defined by the American Psychiatric Association, and the prevalence of brain microbleeds at its different stages, as per Yates et al. (2014).

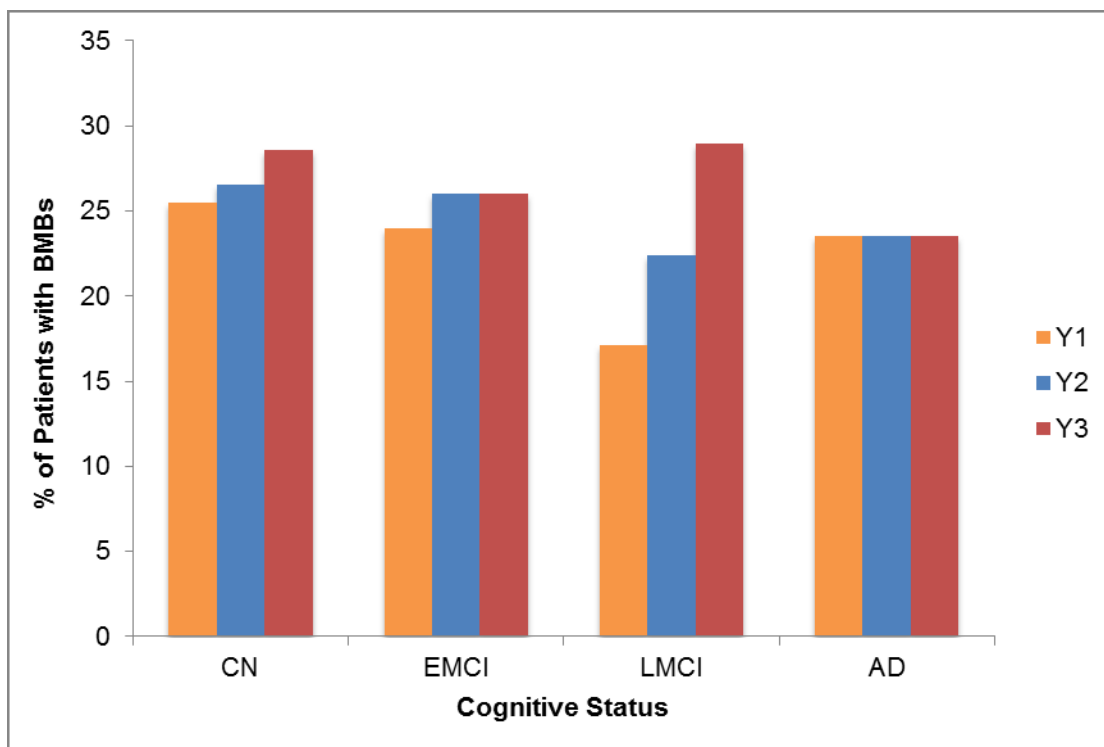
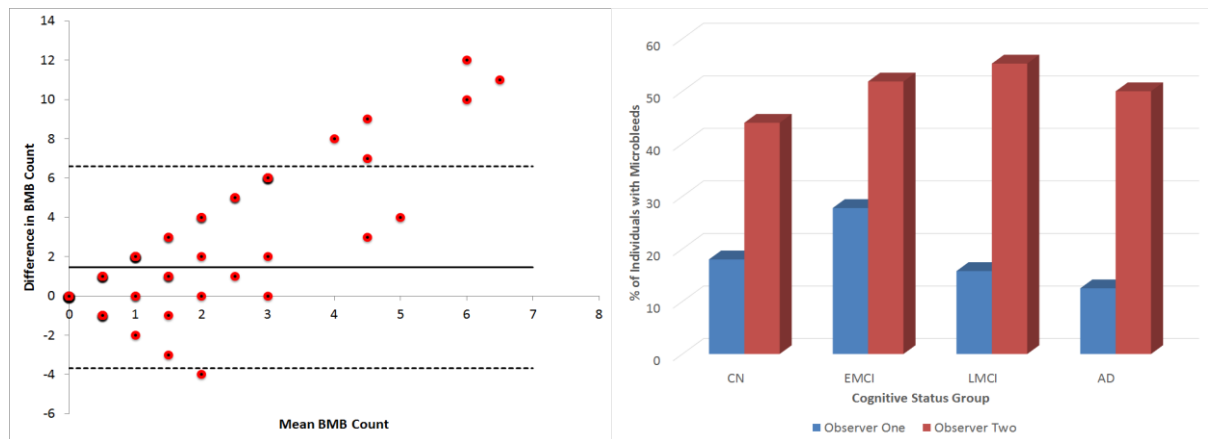


Figure 2. Percentage of patients with BMBs in each cognitive group each year in the total sample of 291 patients. Y1: year 1, Y2: year 2, Y3: year 3, CN: cognitively normal, EMCI: early mild cognitive impairment, LMCI: late mild cognitive impairment, AD: Alzheimer's Disease.



(a)

(b)

Figure 3. Baseline inter-observer variability and BMB prevalence. (a) Bland-Altman plot for BMB count. The difference in BMB count between observers is plotted against the mean BMB count for 150 patients. It increased with the increase of the mean number of BMB counted by both observers. The average difference of BMB count between observers is 1.47, suggesting a bias towards Observer 2 reporting a higher count. Larger circles in the graph represent greater numbers of observer comparisons for these data points. (b) Percentage of patients with BMBs in each cognitive group reported by each observer in the same subsample of 150 patients.

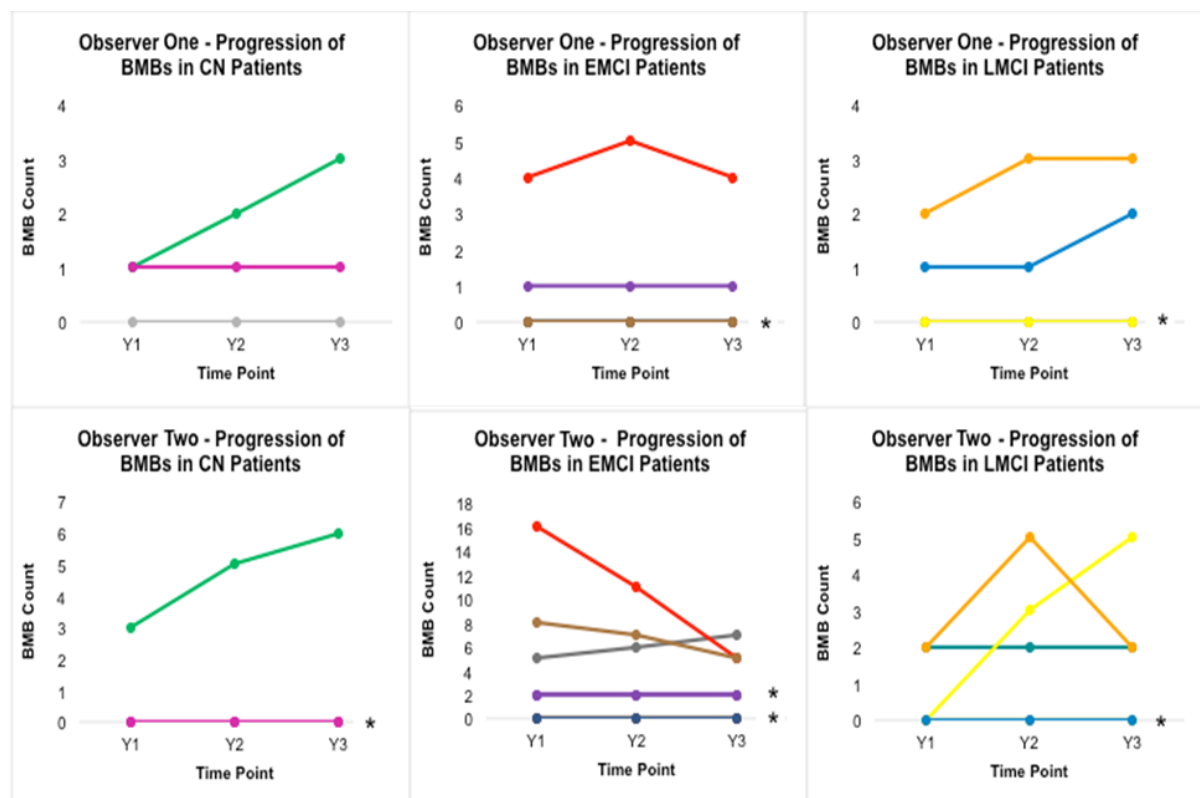


Figure 4. Longitudinal inter-observer differences per cognitive group in 20 patients. Each patient is represented by different colour and the patient colours correspond for both

observers.*: representative of more than one patient. BMB count refers to the number of BMB that each observer counted on each scan.

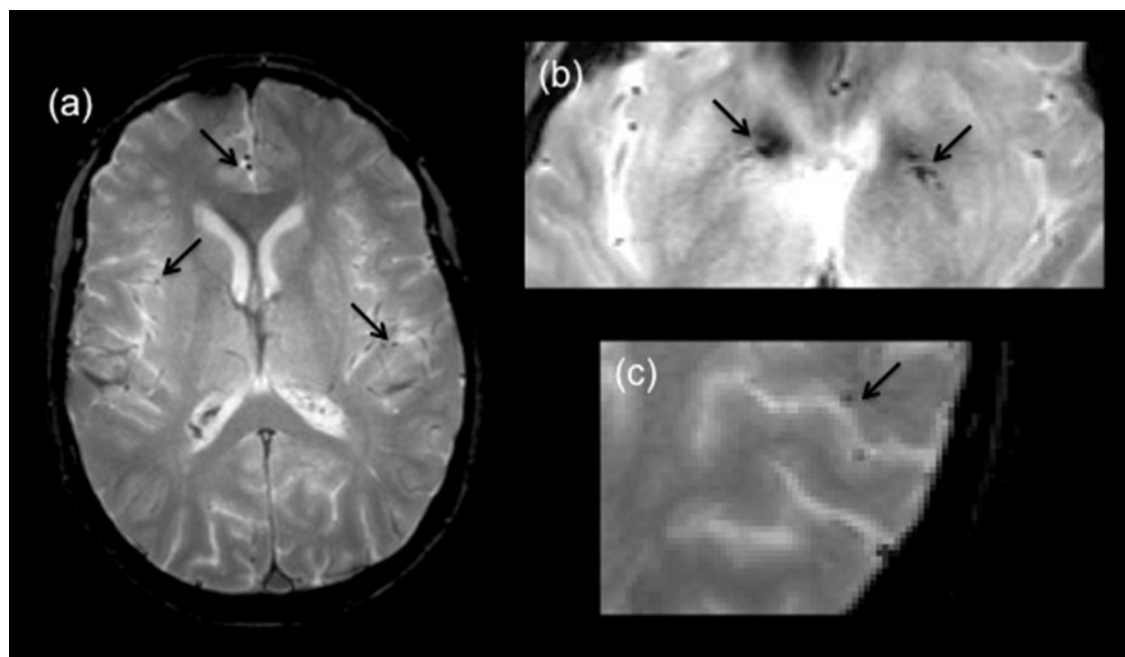


Figure 5. Factors that affected inter-observer variability as seen on MRI T2*-weighted gradient-recalled echo imaging (arrows). (a): vessel calcifications, (b): basal ganglia mineralization, (c): reduced cortical thickness.